BIOLOGIA PLANTARUM 55 (2): 357-360, 2011

BRIEF COMMUNICATION

Overexpression of the *Arabidopsis thaliana* squalene synthase gene in *Withania coagulans* hairy root cultures

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Abstract

Squalene synthase (SS) dimerizes two molecules of farnesyl diphosphate to synthesize squalene, a shared precursor in steroid and triterpenoid biosynthesis in plants. The *SS1* gene encoding SS from *Arabidopsis thaliana* was introduced in *Withania coagulans* under the control of the CaMV35S promoter together with the T-DNA of *Agrobacterium rhizogenes* A4. The engineered hairy roots were studied for withanolide production and phytosterol accumulation and the results were compared with those obtained from control roots harbouring only the T-DNA from pRiA4. The increased capacity of the engineered roots for biosynthesizing phytosterols and withanolides was strongly related with the expression level of the transgene, showing the effectiveness of overexpressing 35*SS1* to increase triterpenoid biosynthesis.

Additional key words: Agrobacterium rhizogenes, phytosterols, *Solanaceae*, withaferin A, withanolide A.

The genus *Withania* (*Solanaceae*) includes two medicinally important species, *i.e.*, *W. somnifera* (L.) Dunal and *W. coagulans* (Stocks) Dunal (Kapoor 2001), whose properties have been attributed to steroidal lactones named withanolides. A recent review of the pharmacological activity of *Withania* constituents (Mirjalili *et al*. 2009b) has highlighted the antitumour activity of withaferin A (WFA) (Bargagna-Mohan *et al*. 2007) and the neuropharmacological properties of withanolide A (WNA) (Tohda *et al*. 2005). An alternative approach to the production of bioactive secondary metabolites is using *in vitro* and metabolic engineering techniques (Palazon *et al*. 2008). The success of *Agrobacterium*-mediated transformation mainly depends on the plant host (Karami *et al*. 2009). A recent transformation of *Withania coagulans* with *A. rhizogenes* produced hairy roots capable of biosynthesising WFA and WNA (Mirjalili *et al*. 2009a).

In *in vitro* cultures, the biosynthesis of withanolides is related with organogenesis (Sharada *et al*. 2007), but only a few aspects of this metabolic pathway are currently known (Sangwan *et al*. 2008, Mirjalili *et al*. 2009b). Squalene synthase (SS, EC2.5.1.21) catalyzes the first step in sterol and triperpenoid biosynthesis, and it could play an important regulatory role in withanolide biosynthesis by acting at a putative key branch point. Overexpression of the *PgSS1* gene in hairy root cultures of *Panax ginseng* (Lee *et al*. 2004) and *Eleutherococcus senticosus* (Seo *et al*. 2005*)* has resulted in an increase of both triterpenoid and phytosterol compounds. Until now the *SS* gene from *Withania* species has not been cloned, so the *SS1* gene from *Arabidopsis thaliana* was used for this study (Kribii *et al*. 1997). To understand the role of SS in phytosterol and withanolide biosynthesis and the competition between both biosynthetic pathways for the share precursor squalene, we investigated the production of both types of compounds in hairy root cultures of *W. coagulans* overexpressing the *AtSS1* gene.

Received 8 August 2009, *accepted* 4 January 2010.
Abbreviations: CaMV - cauliflower mosaic virus; HPLC - high performance liquid chromatography; MS - Murashige and Skoog; PCR - polymerase chain reaction; SS - squalene synthase; WFA - withaferin A; WNA - withanolide A.
Acknowledgements: We thank the Serveis Cientificotècnics of the University of Barcelona for their support and Dr. Boronat's group for the construction of the pBIs *SS1* vector. This research has been supported by grants from the Spanish MEC and Generalitat of Catalonia (BIO2005-05583; BIO2008-01210; 2009 SGR 1217).
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Sterile leaf sections of *Withania coagulans* (Stocks) Dunal from *in vitro* cultures were inoculated with *Agrobacteriun tumefaciens* C58C1 (pRiA4) or *A. tumefaciens* C58C1 (pRiA4) (pBIs *SS1*) carrying the *SS1*cDNA gene. The establishment of the hairy root lines and their culture was carried out as described previously (Mirjalili *et al*. 2009a).

The *rolC* gene was chosen to check the transformed nature of the established root lines. Isolation of total DNA and PCR analysis for *rolC* and *virD1* was carried out as described previously (Moyano *et al*. 1999, Tiwari *et al*. 2008). In the case of the *SS1*cDNA transgene, PCR amplification was performed using the *SS1*-forward primer 5'-TGG GGA GCT TGG GGA CGA TGC-3' and *SS1*-reverse primer 5'-CGG CGT TAC GGA GCT CGG TGT T-3' under the following conditions: initial denaturation at 94 ºC for 5 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 58 ºC for 30 s and extension at 72 ºC for 45 s, with a final extension at 72 ºC for 7 min.

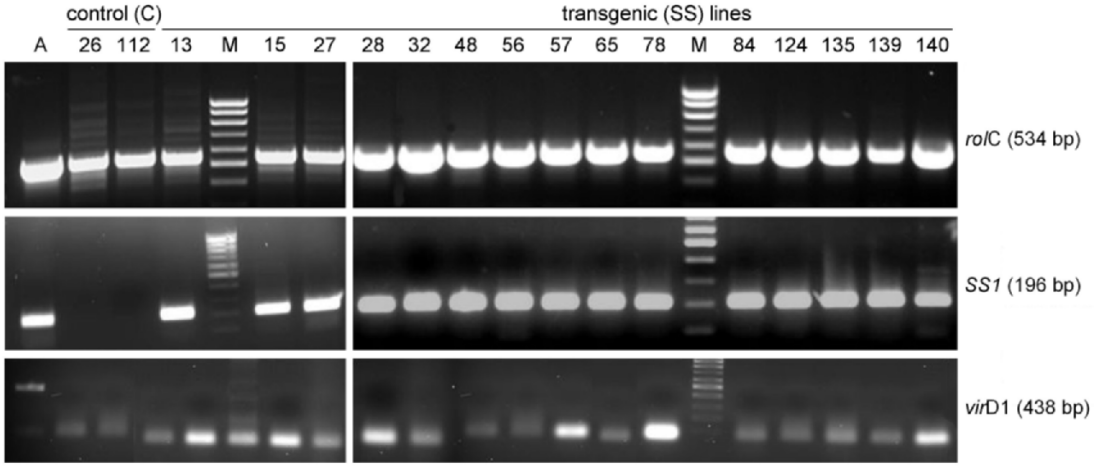

Fig. 1. Representative PCR analyses for the *rol*C, *SS1* and *virD1* genes in transformed root lines. A - *Agrobacterium tumefaciens* strain C58C1 (pRiA4) (pBIs *SS1*); C and SS - control and transgenic hairy root lines, M - 100 bp ladder.

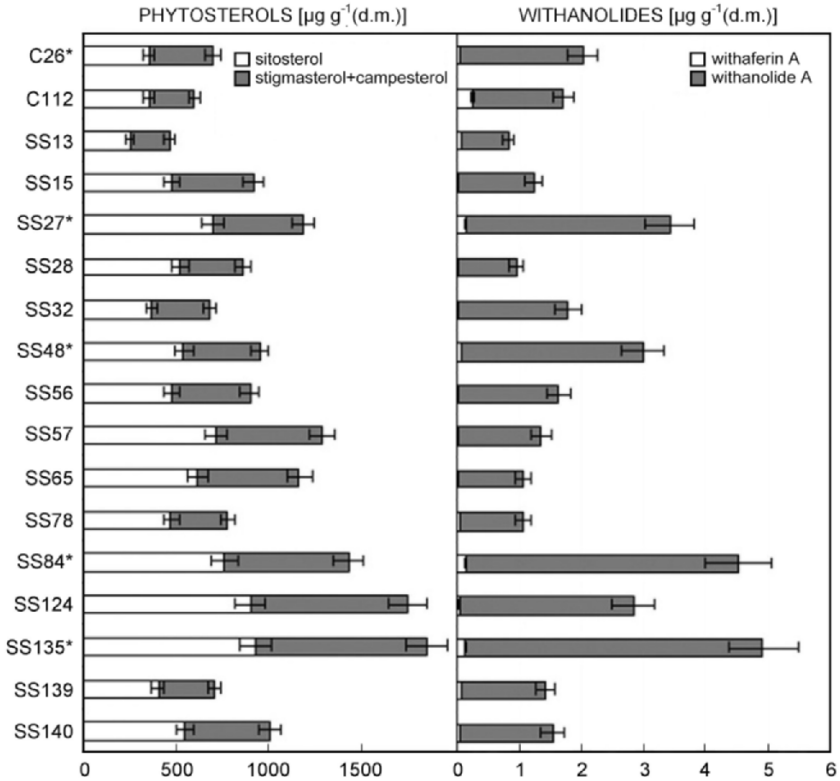

Fig. 2. Steroid and withanolide contents of transformed root lines at the end of the culture period (4 weeks). * - roots with callus-like morphology. Each value is the average of 3 - 4 replicates. *Bars* represent standard deviations.

Duplicates of root samples frozen with liquid nitrogen and stored at -80 ºC were used to check the expression level of squalene synthase and the housekeeping gene *maturase K* (De Keyser *et al*. 2004). Total RNA was extracted and the first strand of cDNA was synthesized as described previously (Mangas *et al*. 2008). PCR was performed using *PuReTag Ready-To-Go* PCR beads (*GE Healthcare*, Buckinghamshire, UK) and was carried out on a programmable thermocycler (*MiniCycler*, *MJ Research*, Watertown, MA, USA). The PCR for *maturase K* was performed using the primers Mat WC-forward 5'-TAC CCT ACC CCG TTC ATC TGG A-3' and Mat WC-reverse 5'-CGT TCA AGA AGG GCT CCA AAA G-3' under the following conditions: initial denaturation at 94 ºC for 5 min, followed by 25 cycles at 94 ºC for 30 s, 61 ºC for 30 s and 72 ºC for 45 s and then a final extension at 72 ºC for 7 min. The PCR for the *SS1*cDNA gene was performed under the above mentioned conditions using 26 cycles. Three replicates for each biological sample were used for reverse transcriptase (RT-PCR) analysis.

Phytosterol analyses were performed as previously described by Mangas *et al*. (2008). For withanolide extraction we followed the method reported by Chaurasiya *et al*. (2008) and HPLC analysis was performed as previously described by Mirjalili *et al*. (2009a).

A. tumefaciens C58C1 carrying pRiA4, alone or together with the plasmid pBIs *SS1,* was able to transform *W. coagulans* plantlets. Hairy roots were formed 3 to 4 weeks after inoculation in over 90 % of the explants. After hairy root emergence, individual root clones were pre-selected in MS solid medium (Murashige and Skoog 1962), and supplemented with 50 mg dm^{-3} kanamycin for at least six generations. Transformed root lines were transferred to MS liquid medium and routinely sub-cultured every four weeks.

The presence of *SS1* and *rolC* genes in the hairy root genome was confirmed by PCR (Fig. 1). We selected the transgenic root lines that gave two bands, the one at 196 bp corresponding to the *AtSS1* gene and the other at 534 bp to the *rolC* fragment from *A. rhizogenes*. The coexistence of both genes confirmed the double transformation of the transgenic roots. The absence of *Agrobacterium* was checked in all the hairy root lines by PCR, amplifying the *virD1* gene of *A. rhizogenes* (Fig. 1).

Like the control roots, transgenic roots carrying the *SS1* gene (SS lines) displayed typical hairy root and callus-like morphology, and a variable growth capacity. Both types of morphologies and similar variations in growth capacity have been previously described for wild-type hairy root lines of *W. coagulans* (Mirjalili *et al*. 2009a) and *W. somnifera* (Bandyopadhyay *et al*. 2007).

SS lines displayed a similar steroid pattern to that of the controls, generally showing a higher content of sitosterol than stigmasterol + campesterol. Although there were significant differences among root lines, the average total phytosterol content was significantly higher ($P < 0.01$) in the SS lines than in the control (Fig. 2). This result matches an earlier report for *Panax ginseng* overexpressing *PgSS1* (Lee *et al*. 2004). The phytosterol contents were particularly high in lines SS124, with typical hairy root morphology, and SS135, with callus-like morphology, which achieved a total content of 1.75 and 1.85 mg g^{-1}(d.m.), respectively, by the end of the culture period.

As in the control lines, WNA was an abundant compound in the withanolide spectrum of SS transgenic root lines, while WFA was quantified in only small

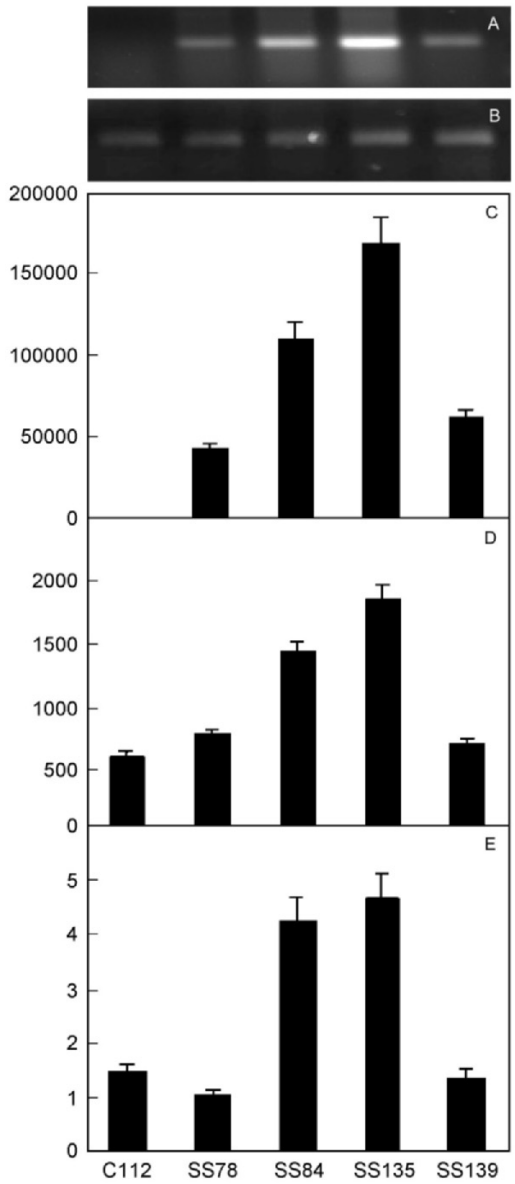

Fig. 3. Analysis of *SS1* gene expression. *A* - RT-PCR analysis of the *SS1* gene. Control C112 and transgenic lines SS78, SS84, SS135, SS139. *B* - The *maturase K* (*matK*) gene was used as a loading control. *C* - Intensities of the *SS1* amplicon measured using *Kodak* molecular imaging software. *D* - Total steroid contents [μg g^{-1}(d.m.)]. *E* - Total withanolide contents [μg g^{-1}(d.m.)]. All the values are the means of 3 - 4 replicates. *Bars* represent standard deviations.

quantities (Fig. 2). These results also match those previously reported for *W. somnifera* hairy roots, all of which have shown WNA (Murthy *et al*. 2008) with only a sporadic detection of WFA in some of the established root lines (Bandyopadhyay *et al*. 2007). The WNA content accumulated in *W. coagulans* transgenic roots varied widely, ranging from 0.68 (line SS13) to 4.63 μg g^{-1}(d.m.) (line SS135). This was significantly higher than in the roots of the *in vitro* plants used as the explant source, which showed a withanolide content of about 0.11 μg g^{-1}(d.m.) after 4 weeks of growth. The engineered root lines were divided into two groups according to their withanolide production: high- (lines SS27, SS48, SS84, SS124, SS135) and low-performing (lines SS13, SS15, SS28, SS32, SS56, SS57, SS65, SS78, SS139, SS140). Overexpression of the 35S-*SS1* transgene significantly increased ($P < 0.01$) withanolide contents only in the former group, in comparison with the most productive control.

To establish a possible relationship between the constitutive *SS1* overexpression and the capacity of the engineered roots to synthesize phytosterol and withanolides, *SS1* gene transcript levels were studied in both control and SS-transgenic root lines. Two highly productive root lines were compared with two root lines with a limited capacity to biosynthesize these compounds. It was shown that the *AtSS1* gene was transcribed in all the SS hairy root lines, but with varying richness (Fig. 3). The strongest *SS1* expression was observed in the highly productive root lines (SS84 and SS135) and lowest expression in the lines with a low production (SS78 and SS139). Combining the results of phytosterol and withanolide production (Fig. 3), it becomes clear that the expression of the 35S-*SS1* gene correlated positively with steroid and withanolide metabolism, confirming the pivotal role of the *SS1* gene in the regulation of primary and secondary triterpene metabolism.

References

Bandyopadhyay, M., Jha, S., Tepfer, D.: Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *Withania somnifera*. - Plant Cell Rep. **26**: 599-609, 2007.

Bargagna-Mohan, P., Hamza, A., Kim, Y., Ho, Y.K., Mor-Vaknin, N., Wendschlag, N., Liu, J., Evans, R., Markovitz, D., Zhan, C.: The tumor inhibitor and antiangiogenic agent withaferin A targets the intermediate filament protein vimentin. - Cell. Press **14**: 623-634, 2007.

Chaurasiya, N.D., Uniyal, G.C., Lal, P., Misra, L., Sangwan, N.S., Tuli, R., Sangwan, R.S.: Analysis of withanolides in root and leaf of *Withania somnifera* by HPLC with photodiode array and evaporative light scattering detection. - Phytochem. Anal. **19**: 148-154, 2008.

De Keyser, E., De Riek, J., Van Bockstaele, E.: Optimization of relative quantitative RT-PCR for expression analysis in azalea flowers colour spots. - Acta Hort. **651**: 91-96, 2004.

Kapoor, L.D.: Handbook of Ayurvedic Medicinal Plants. - CRC Press, London 2001.

Karami, O., Esna-Ashari, M., Karami Kurdistani, G., Aghavaisi, B.: *Agrobacterium*-mediated genetic transformation of plants: the role of host. - Biol. Plant. **53**: 201-212, 2009.

Kribii, R., Arró, M., Del Arco, A., González, V., Balcells, L., Delourme, D., Ferrer, A., Karst, F., Boronat, A.: Cloning and characterization of the *Arabidopsis thaliana* SQS1 gene encoding squalene synthase. - Eur. J. Biochem. **249**: 61-69, 1997.

Lee, M.H., Jeong, J.H., Seo, J.W., Shin, C.G., Kim, Y.S., In, J.G., Yang, D.C., Yi, J.S., Choi, Y.E.: Enhanced triterpene and phytosterol biosynthesis in *Panax ginseng* overexpressing squalene synthase gene. - Plant Cell Physiol. **45**: 976-984, 2004.

Mangas, S., Moyano, E., Osuna, L., Cusido, R.M., Bonfill, M., Palazon, J.: Triterpenoid saponin content and the expression level of some related genes in calli of *Centella asiatica*. - Biotechnol. Lett. **30**: 1853-1859, 2008.

Mirjalili, M.H., Fakhr-Tabatabaei, S.M., Bonfill, M., Alizadeh, H., Cusido, R.M., Ghassempour, A., Palazon, J.: Morphology and withanolide production of *Withania coagulans* hairy root cultures. - Eng. Life. Sci. **9**: 197-204, 2009a.

Mirjalili, M.H., Moyano, E., Bonfill, M., Cusido, R.M., Palazon, J.: Streoidal lactones from *Withania somnifera*, an ancient plant for novel medicine. - Molecules **14**: 2373-2393, 2009b.

Moyano, E., Fornalé, S., Palazon, J., Cusidó, R.M., Bonfill, M., Morales, C., Piñol, M.T.: Effect of *Agrobacterium rhizogenes* T-DNA on alkaloid production in Solanaceae plants. - Phytochemistry **52**: 1287-1292, 1999.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant **15**: 473-497, 1962.

Murthy, H.N., Dijkstra, C., Anthony, P., White, D.A., Davey, M.R., Power, J.B., Hahn, E.J., Paek, K.Y.: Establishment of *Withania somnifera* hairy root cultures for the production of withanolide A. - J. Integr. Plant Biol. **50**: 975-981, 2008.

Palazon, J., Navarro-Ocaña, A., Hernandez-Vazquez, L., Mirjalili, M.H.: Application of metabolic engineering to the production of scopolamine. - Molecules **13**: 1722-1742, 2008.

Sangwan, R.S., Chaurasiya, N.D., Lal, P., Misra, L., Tuli, R., Sangwan, N.S.: Withanolide A is inherently *de novo* biosynthesized in roots of the medicinal plant ashwagandha (*Withania somnifera*). - Physiol. Plant **133**: 278-287, 2008.

Seo, J.W., Jeong, J.H., Shin, C.H., Lo, S.C., Han, S.S., Yu, K.W., Harada, E., Han, J.Y., Jeong, Y., Choi, Y.E.: Overexpression of squalene synthase in *Eleutherococcus senticosus* increases phytosterol and triterpene accumulation. - Phytochemistry **66**: 869-877, 2005.

Sharada M., Ahuja, A., Suri, K.A., Vij, S.P., Khajuria, R.K., Verma, V., Kumar, A.: Withanolide production by *in vitro* cultures of *Withania somnifera* and its association with differentiation. - Biol. Plant. **51**: 161-164, 2007.

Tiwari, R.K., Trivedi, M., Guang, Z.C., Guo, G.Q., Zhen, G.C.: *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. - Biol. Plant. **52**: 26.35, 2008.

Tohda, C., Komatsu, K., Kuboyama, T.J.: Scientific basis for the anti-dementia drugs of constituents from Ashwagandha (*Withania somnifera*). - J. Tradit. Med. **22**: 176-182, 2005.